Proteomic analysis of ovaries of queen bees (*Apis mellifera*) developed from larvae grafted at different instars

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Abstract: 【Aim】 To explore the molecular regulation mechanism of grafted larvae on ovarian development of queens in the Western honey bee, *Apis mellifera*. 【Methods】 The isobaric tags for relative and absolute quantitation (iTRAQ) method was used to quantify the proteins in the ovaries of queens reared from the larvae grafted at different instars and screen the differentially expressed proteins (DEPs). The results were validated by Western blotting. 【Results】 A total of 452 966 spectra were obtained and 3 642 proteins were identified from ovaries of *A. mellifera* queens. Gene Ontology (GO) analysis revealed that the DEPs in the ovaries of queens reared from the larvae grafted at different instars are associated with cell metabolism, cell division, and protein biosynthesis. The DEPs in the ovaries of queens reared from the larvae grafted between the 1st and 2nd instars are enriched in such pathways as carbohydrate metabolism, lipid metabolism, and exogenous degradation, while those reared from the larvae grafted between the 1st and 3rd instars mainly enriched in developmental pathways, ribosome pathways, and lysosome metabolism. The Western blotting results of the two differentially expressed storage proteins hexamerin 110 and hexamerin 70b showed that their expression levels decreased with the grafted larval instar. 【Conclusion】 The identification of differentially expressed proteins in the ovaries of *A. mellifera* queens reared from the larvae grafted at different instars provides a foundation for the regulatory mechanism of queen reproductive development and caste differentiation.

Key words: *Apis mellifera*; grafted larva; instar; ovary; queen; proteomics; differentially expressed proteins

1 INTRODUCTION

The honeybees are social insects that live together as a unit with one fertile female (the queen), several thousand sterile females (the workers), and a few hundred male drones. Queen and worker bees all originate from the zygote; they have the same genetic composition, but differ in external form, physiological structure, and behavior. Queens are larger, live much longer, have specialized anatomy, and develop faster from egg to adult. However, the worker bees are smaller, have a shorter life-span, and cannot reproduce (Hartfelder and Engels, 1998; Page and Peng, 2001), this phenomenon is called caste differentiation and has become a research hot topic at present. Shi (2011) and Chen and Hu (2011) demonstrated that food intake determines which larvae will develop into queen and worker bees. Whole genome sequencing was completed for *Apis mellifera* in 2006, many in-depth studies on the mechanism of bee class differentiation at the gene and protein levels have since been conducted (Evans and Wheeler, 2000; Barchuk *et al.*, 2007; Li *et al.*, 2010; Wu and Li, 2010; Chen *et al.*, 2012), but studies on ovaries of honeybees at the proteome level are scarce.

The ovary is an important organ in female bees, and its size and development are indicative of the queen's fecundity. Differences in reproductive ability between the queen and worker bees are mainly because 95% ovarian degradation occurs in the workers (Capella and Hartfelder, 1998). Previous studies have shown that the total sugar (glucose, fructose and sucrose) content of royal jelly, which was fed to queen larvae, was higher than that in the worker jelly (Lensky, 1985; Severson *et al.*, 1989); sugar stimulates larval bees to feed and plays a role in accelerating development (Asencot and Lensky, 1984). Furthermore, the composition of worker jelly differed from that of royal jelly, except

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for saccharinity (Severson *et al.*, 1989). The nutritional components and content of royal jelly were significantly higher than those of Wangtaipu royal jelly, therefore, the queen larvae grafted earlier ate royal jelly for longer. The nutritional differences between worker and queen at their larval stage affected their development (Kucharski *et al.*, 2008). As early as 400 years ago, food began to determine if larvae developed into either queen or worker bees (Shi, 2011; Chen and Hu, 2011), which resulted in the development of different castes (Haydak, 1943; Allsopp *et al.*, 2003). During artificial queen cultivation, the instar of the grafted larvae impacts the queen development, with the late instars having greater influence on the two-way development ability of larva (Shen *et al.*, 2013), and the earlier instars having less effect. Therefore, during artificial queen cultivation, it is crucial to determine the best time to graft larvae.

Woyke (1971) and Weaver (1957) reported that older grafted instar larvae had fewer ovarian tubes. Ali *et al.* (2014) measured the productivity of *Apis mellifera* queens reared from the larvae grafted at the 1st, 2nd, and 3rd instars, the results revealed that the instar affected queen productivity. During artificial queen cultivation, the instar of the grafted larvae has an influence on the queen development. In previous proteomic researches on honeybee, 2-dimensional differential gel electrophoresis (2-DE) technique was mainly used for protein identification (Li *et al.*, 2008; Feng *et al.*, 2009). Though the electrophoretic approach is widely used, it is prone to experimental errors (Righetti *et al.*, 2004). The isobaric tags for relative and absolute quantitation (iTRAQ) method can enhance the sensitivity of screening proteome (Mertins *et al.*, 2012), and has been widely employed since its invention (Treumann and Thiede, 2010). To clarify the molecular mechanism that the grafted larval instar regulates the queen growth and development, we analyzed the proteomics of ovaries of *A. mellifera* queens reared from the larvae grafted at different instars using the iTRAQ method, and identified a significant number of differentially expressed proteins (DEPs) among the groups grafted at the 1st, 2nd and 3rd larval instars. The findings provided new insights into the influences of different instars grafted larvae on queen bee development.

2 MATERIALS AND METHODS

2.1 Preparation of protein samples

The virgin queens (*A. mellifera*) emerged and single drone sperm was artificially injected 8 days later; the honeybee colonies with inseminated queens were organized to lay eggs. The *A. mellifera* colonies were raised in an animal yard in natural conditions at the Apiculture Science Institute of Jilin Province, China.

Sterilized empty combs were put into the oviposition limiting tools. After being cleaned for 24 h by workers, the queens were allowed to lay eggs for 6 h, the empty combs were then transferred into a super containing many worker bees to hatch. After 98 h (1st instar), 122 h (2nd instar), and 146 h (3rd instar), respectively, the larvae were collected and bred until the day before the queens emerged from the cocoon. The larvae were all transferred to the incubator until queens emerged. The ovaries of newly emerged queens were collected, immediately preserved in liquid nitrogen, and stored at −80℃ for further use.

2.2 Protein isolation and labeling

The queen ovaries were pooled (n = 16) as one sample and grinded to powder in liquid nitrogen. The powder was put into 1.5 mL tubes, 1 mL cold acetone was added first, and 10 mmol/L dithiothreitol (DTT) was then added. The protein extraction was carried out by reference to the method of Ji *et al.* (2014). The protein concentration was determined according to Bradford method (Bradford, 1976). Each sample was heated for 5 min at 95℃, then immediately suspended into 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) running buffer, and vortexed prior to gel electrophoresis. The gels were run at 120 V for 120 min.

2.3 Tryptic digestion and iTRAQ

One hundred μg protein from each sample was mixed with trypsin in a ratio of 20∶1 (sample∶trypsin, v/v), and then incubated for 4 h at 37℃. After that, trypsin was added again in the same proportion to continue incubating for 8 h.

After tryptic digestion, the peptides were dried by vacuum pump, and 0.5 mol/L tetraethylammonium bromide (TEAB) was then added to the dissolved peptides. Every group of peptides was labeled with iTRAQ reagents and incubated at room temperature for 2 h.

2.4 SCX separation

The iTRAQ labeled peptides were separated in an Ultremex strong cation exchange Choematography (SCX) (Shimadzu LC-20AB liquid phase system, 4.6 mm × 250 mm specification) separation column. The dried peptides with iTRAQ labeled were repeatedly dissolved in 4 mL buffer A [25 mmol/L NaH_2PO in 25% acetonitrile (ACN), pH 2.7].

The peptides were then eluted for 22 min with increasing buffer B (25 mmol/L NaH_2PO_4, 1 mol/L KCl in 25% ACN, pH 2.7) concentrations from 5% to 80% (v/v) with a flow of 1 mL/min, and the absorbance was 214 nm. Every component was desalted in a StrataX desalting column and then freeze-dried.

2.5 Identification of proteins using LC-ESI-MS/MS

The 10 μL peptides were subjected to LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) analysis via a nanoelectrospray III ion source (AB SCIEX, ON, CA; spray voltage 2.5 kV, spray seam temperature 150℃). The peptides were demineralized and adsorbed on the Symmetry C18 Trap column (5 μm, 180 μm × 20 mm, Thermo Fisher Scientific) in the loading solvent (0.1% formic acid, 2% acetonitrile in H_2O) with a flow of 2 μL/min for 15 min. The peptides were separated on the BEH130 C18 column (1.7 μm, 100 μm × 100 mm, Thermo Fisher Scientific) using a 50-min elution with a 5% – 80% acetonitrile (0.1% formic acid) gradient at 300 nL/min.

Precursor ion selection was made on the basis of ion intensity (peptide signal intensity above 120 counts/s) and charge state (2 + to 5 +). Each scan signal was recorded separately with four channels, a total of four times, and finally merged into the data. MS data acquisition was carried out in a data-dependent manner. The fracture energy ion was 35 ± 5 eV. Dynamic exclusion was enabled with a repeat count of no more than twice during the first half of peak outflow (approximately 18 s).

2.6 Database search and quantification

The output file data (*.wiff) was converted to *.mgf format and submitted to the reference data, and searches were conducted using the Mascot search engine version 2.3.02. The tolerance of the MS/MS ion search contracting type and fragment was set to ± 0.1 D, peptide error tolerances were set to ± 0.05 D (Noirel *et al.*, 2011). The proteins with ratios that differed by >1.5 times ($P < 0.05$) were regarded as differentially expressed proteins.

2.7 Bioinformatics analysis

The functional gene ontology (GO) category was used to assign the proteins identified into specific GO terms. The Kyoto Encyclopedia of Genes and Genomes (KEGG) based annotation system was used to analyze the statistically significant biological pathways of the proteins identified.

2.8 Validation by Western blotting

To validate the data, the expression levels of differentially expressed proteins were examined by Western blotting. Two proteins, hexamerin 110 and hexamerin 70b, were closely related with caste differentiation. The antibodies were prepared by Abmart (http://www.abcam.com). The monoclonal antibodies were anti-hexamerin 110 protein and anti-hexamerin 70b protein at dilutions of 1∶1 000. The secondary antibody was horseradish peroxidase-conjugated goat anti-rat at a dilution of 1∶10 000. β-actin was used as a reference protein for normalization.

3 RESULTS

3.1 Protein identification results

A total of 452 966 second-order spectra, 55 600 matching spectra, 53 239 unique spectra, 22 423 peptides, and 21 958 unique peptides were identified in ovaries of *A. mellifera* queen; 3 642 proteins were identified in total. Among these, 933 (25.64%) proteins had a molecular weight >100 kD, while the remaining 2 508 proteins, or half of the proteins, had a molecular weight ranging from 20 to 80 kD.

3.2 Peptide fragment evaluation

The peptide sequence length distribution descriptive statistics revealed that most of them are 7 to 17 amino acids long. Most of the identified proteins contain less than 10 peptides, and the number of proteins decreased with an increase of the number of peptides. The proteins with 0 – 10% coverage accounted for 48% of the total amount, while those with >35% coverage accounted for 13% (Fig. 1).

3.3 Protein expression analysis

3.3.1 Venn diagram analysis: 424 differentially expressed proteins (DEPs) were identified when comparing the 1st instar group to the 2nd instar group. Similarly, 305 and 335 DEPs were obtained when comparing the 1st instar and the 2nd instar groups to the 3rd instar group, respectively (Fig. 2). There were 247 DEPs exclusively expressed in the ovaries of queens between the 1st and 2nd instar grafted larvae groups, 114 DEPs exclusively expressed in the ovaries of queens between the 1st and 3rd instar grafted larvae groups, and 155 DEPs exclusively expressed in the ovaries of queens between the 2nd and 3rd instar grafted larvae groups. There were 38 DEPs common to the three groups, these proteins may be the essential developmental proteins in queen bees (Fig. 3).

3.3.2 GO category enrichment analysis: GO category was used to analyze the differentially expressed proteins in the ovaries of queen reared from the larvae grafted at different instars (Figs. 4 – 6). The major DEPs are associated with basal metabolism (23.9%), single

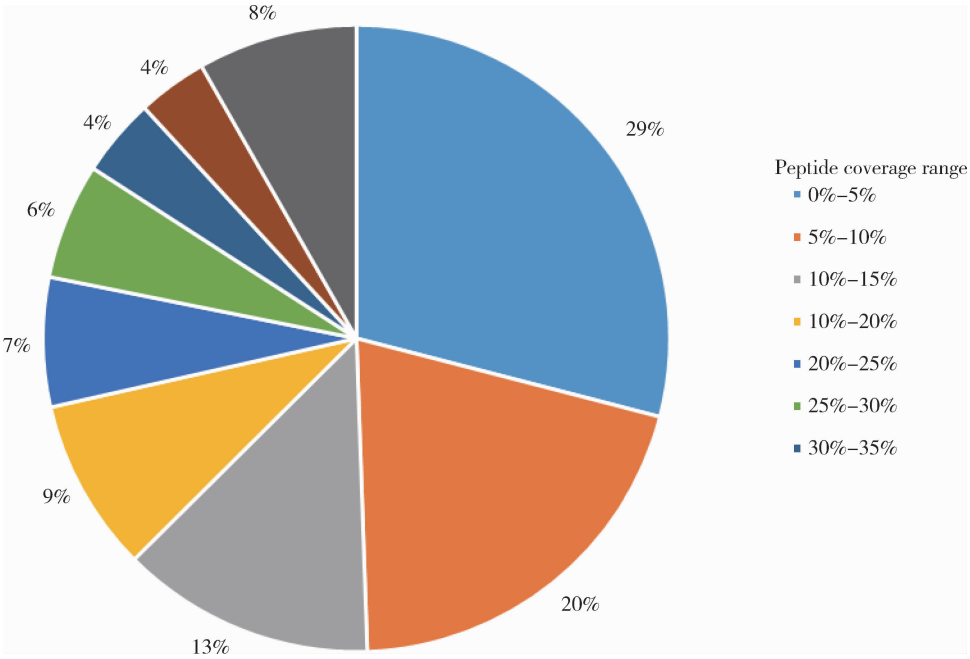

Fig. 1　Sequence coverage distribution of proteins in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

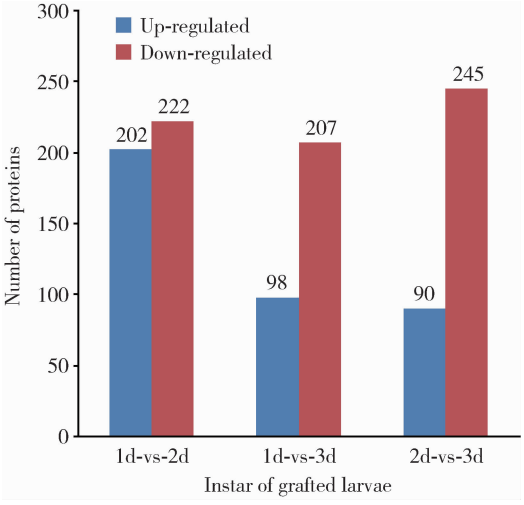

Fig. 2　Distribution and expression profiles of differentially expressed proteins (DEPs) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

The *x*-axis shows the pairwise comparisons of the three sample groups: 1d-vs-2d: DEPs in the ovaries of queens between the 1st and 2nd instar grafted larvae groups; 1d-vs-3d: DEPs in the ovaries of queens between the 1st and 3rd instar grafted larvae groups; 2d-vs-3d: DEPs in the ovaries of queens between the 2nd and 3rd instar grafted larvae groups. 1d: Queens reared from the 1st instar grafted larvae; 2d: Queens reared from the 2nd instar grafted larvae; 3d: Queens reared from the 3rd instar grafted larvae. The same for Figs. 3 – 9.

biological metabolic process (28.7%), cytoplasm (53.5%), cytomembrane (41.4%), ion binding (35.7%), macromolecular compounds (50.5%), and protein complex (36.0%).

3.3.3　Pathway enrichment analysis of DEPs: A total of 424 differentially expressed proteins were identified between the 1st instar and the 2nd instar groups, of which 207 DEPs were enriched. Among 17 enriched pathways, the metabolic pathway contained the most enriched DEPs, followed by the focal adhesion pathways (Fig. 7).

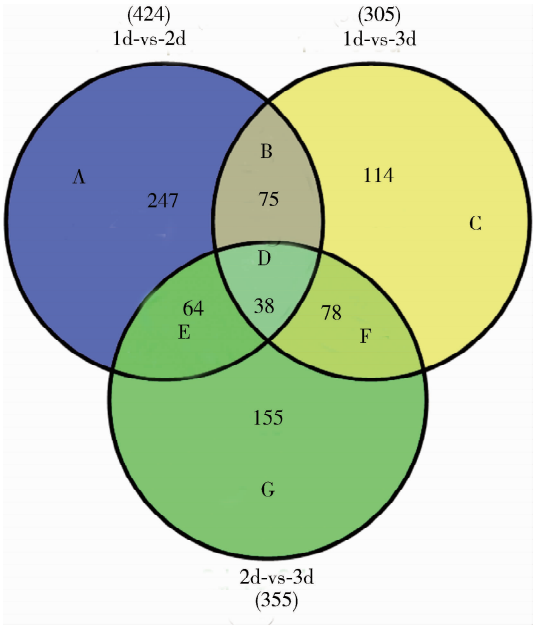

Fig. 3 Venn diagram of differentially expressed proteins (DEPs) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

Venn analysis of DEPs in three samples. The numbers denote the amount of proteins that were expressed in each class. Classes are labeled A – G: Exclusively expressed in group 1d-vs-2d, only expressed in groups 1d-vs-2d and 1d-vs-3d, exclusively expressed in groups 1d-vs-3d, expressed in common, only expressed in groups 1d-vs-2d and 2d-vs-3d, only expressed in groups 1d-vs-3d and 2d-vs-3d, and exclusively expressed in group 2d-vs-3d, respectively.

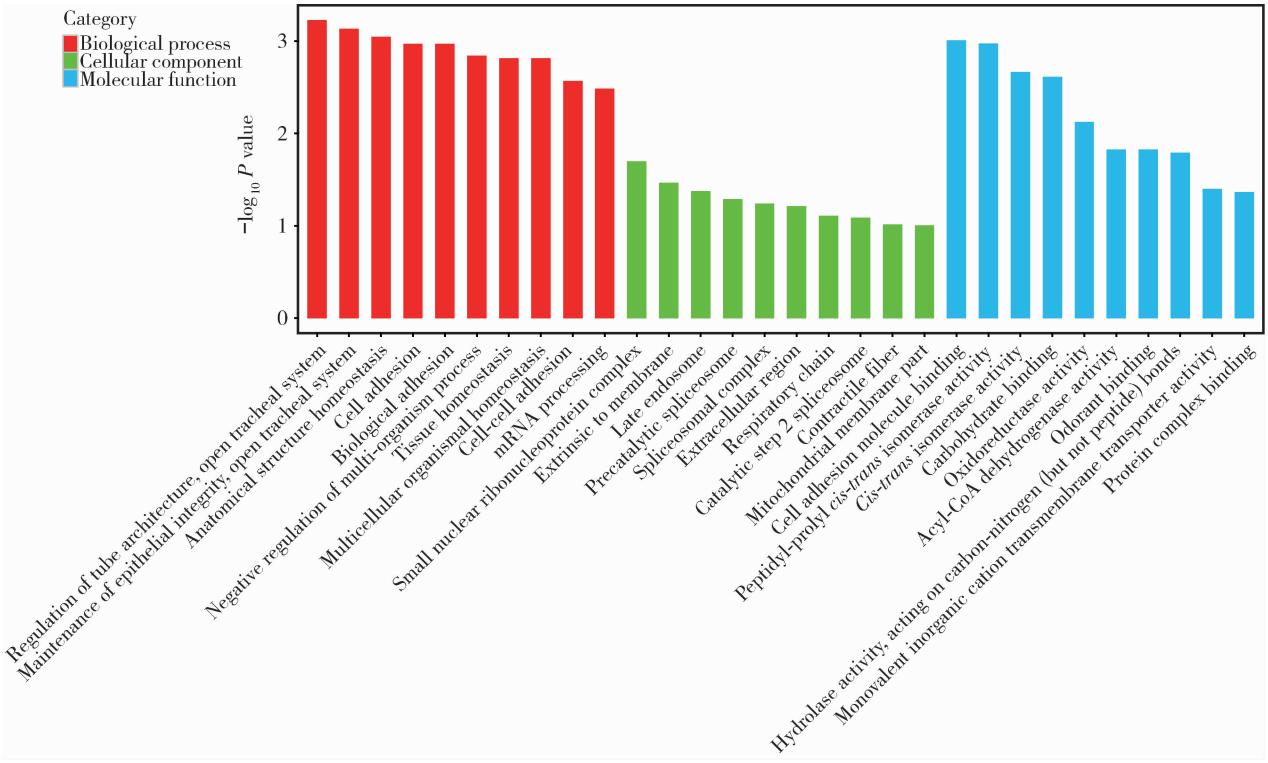

Fig. 4 GO enrichment of DEPs (1st instar *vs* 2nd instar) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

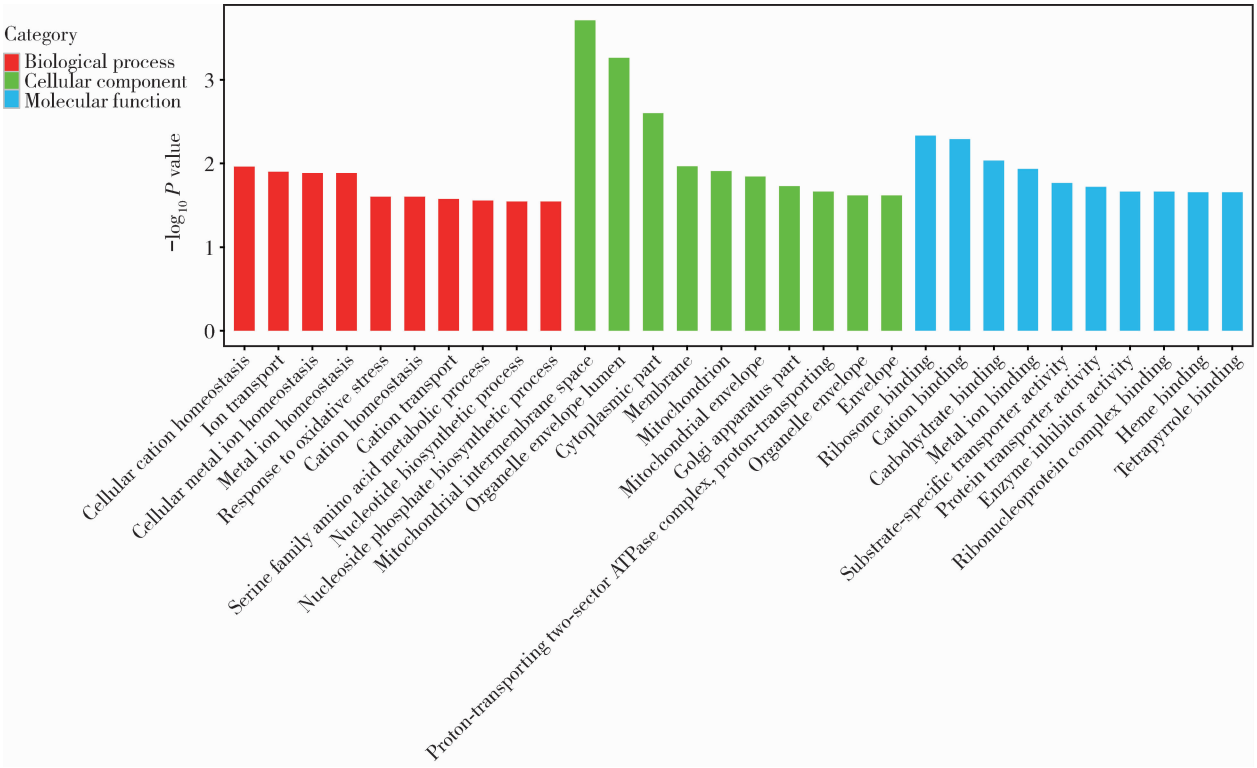

Fig. 5　GO enrichment of DEPs (1st instar *vs* 3rd instar) in the ovaries of *Apis mellifera* queens reared from larvae grafted at different instars

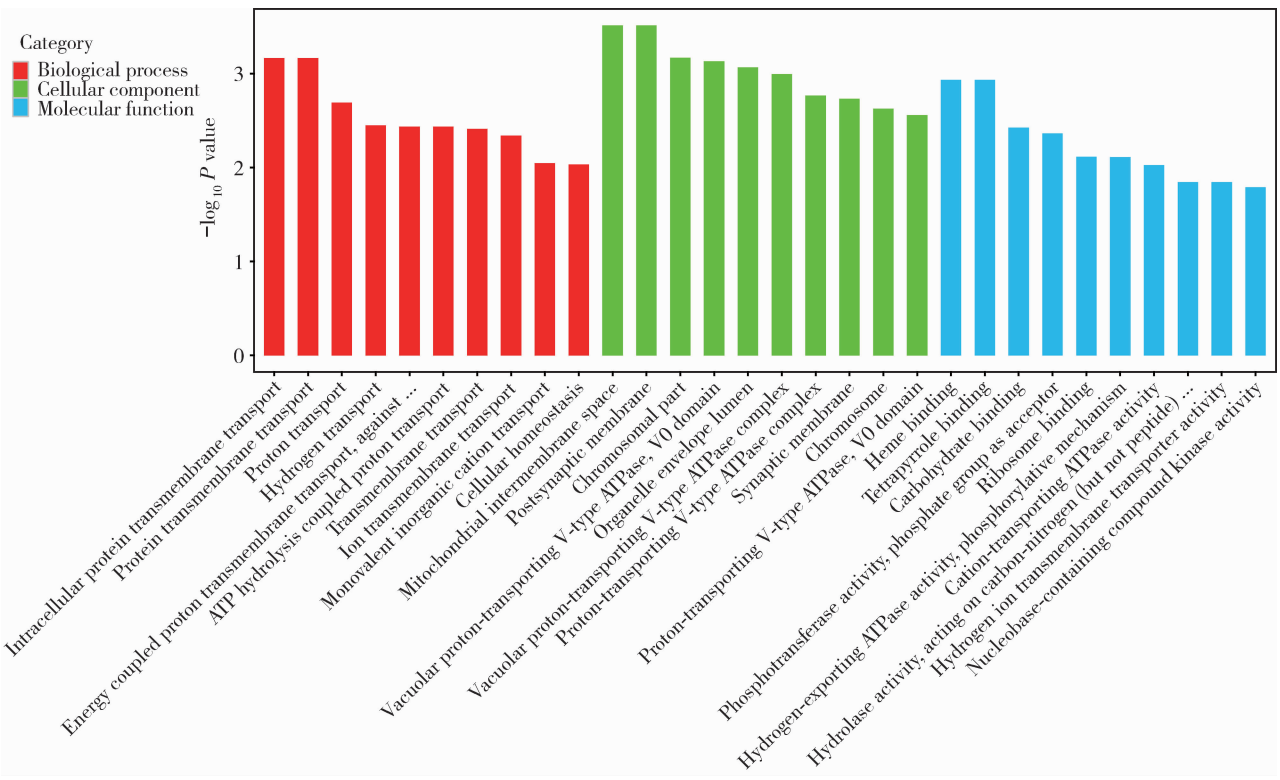

Fig. 6　GO enrichment of DEPs (2nd instar *vs* 3rd instar) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

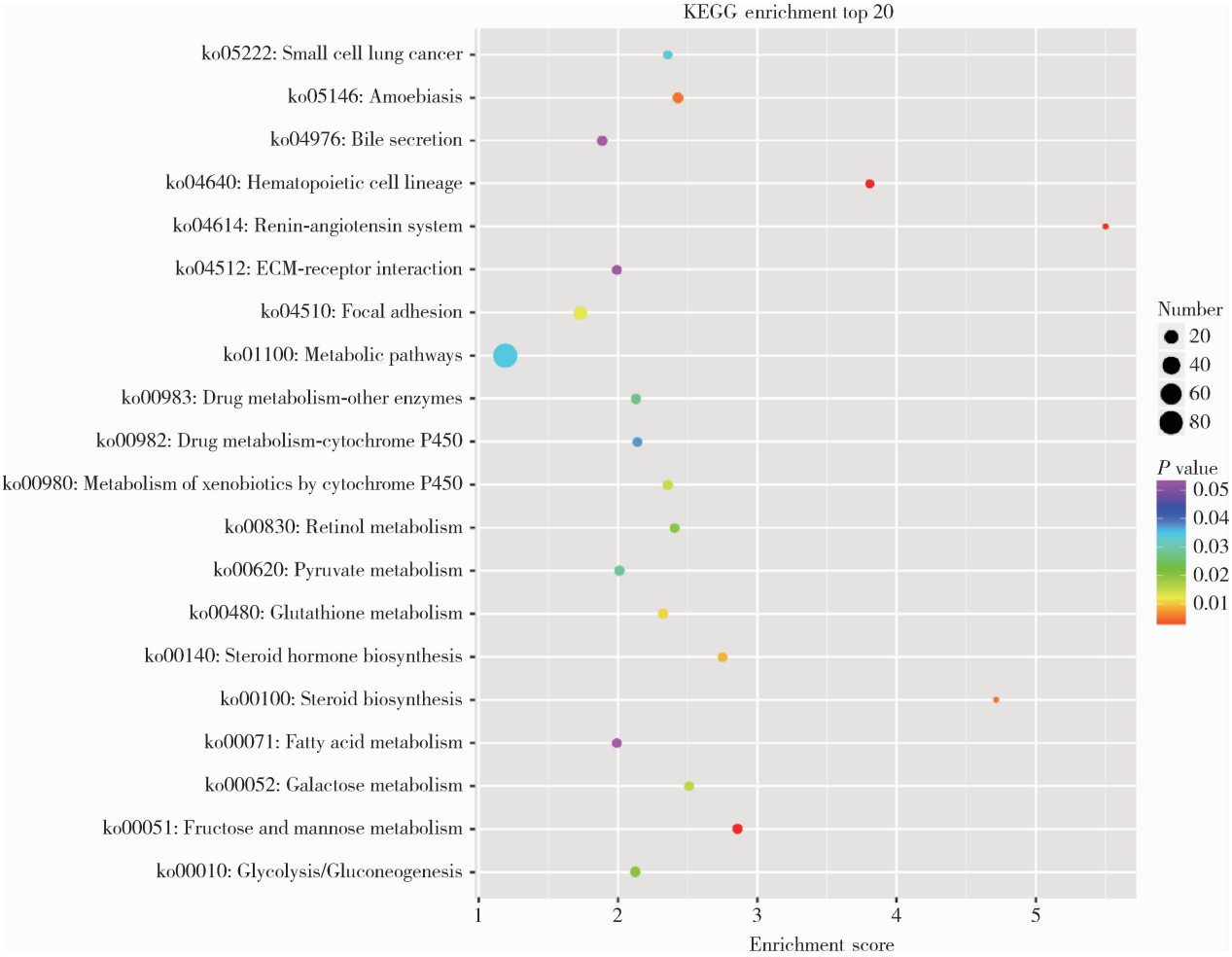

Fig. 7 Enriched DEPs pathways (1st instar *vs* 2nd instar) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

Similarly, there were 305 differentially expressed proteins identified between the 1st and the 3rd instar, of which 181 were enriched. Among the 11 enriched pathways, the ribosome pathway was the most enriched, followed by the lysosome pathway (Fig. 8). There were 335 differentially expressed proteins identified between the 2nd and the 3rd instar, of which 172 were enriched. Among the 17 enriched pathways, the oxidative phosphorylation and Huntington's disease pathways were the most enriched, followed by the paralysis agitans pathway (Fig. 9).

3.4 Verification of protein expression by Western blotting

Among the DEPs, the expression levels of two storage proteins hexamerin 110 and hexamerin 70b decreased with the grafted larval instar as verified by Western blot analysis (Fig. 10). The results were identical with the iTRAQ data.

4 DISCUSSION

Many of the differentially expressed proteins identified in this research were linked to carbohydrate metabolism and protein metabolism. This may be because energy metabolism had an influence on queen development, and larval development needs large amounts of energy and proteins to stimulate enhanced metabolic activities. As shown in Figs. 4 – 6, many DEPs were associated with amino acid metabolism, amino acids have greatly contributed to the changes in larval development (Evans and Wheeler, 2000). Because the ovarian development of queen was similar at different ages, more than 90% metabolic pathways were consistent.

The DEPs in groups grafted at the 1st and 2nd larval instars were enriched in the metabolic pathways (24.17%), including carbohydrate metabolism, lipid metabolism, and exogenous degradation, suggesting that there were differences in growth and metabolic rate between the two groups. The DEPs in groups grafted at the 1st and 3rd larval instars were mainly enriched in the developmental

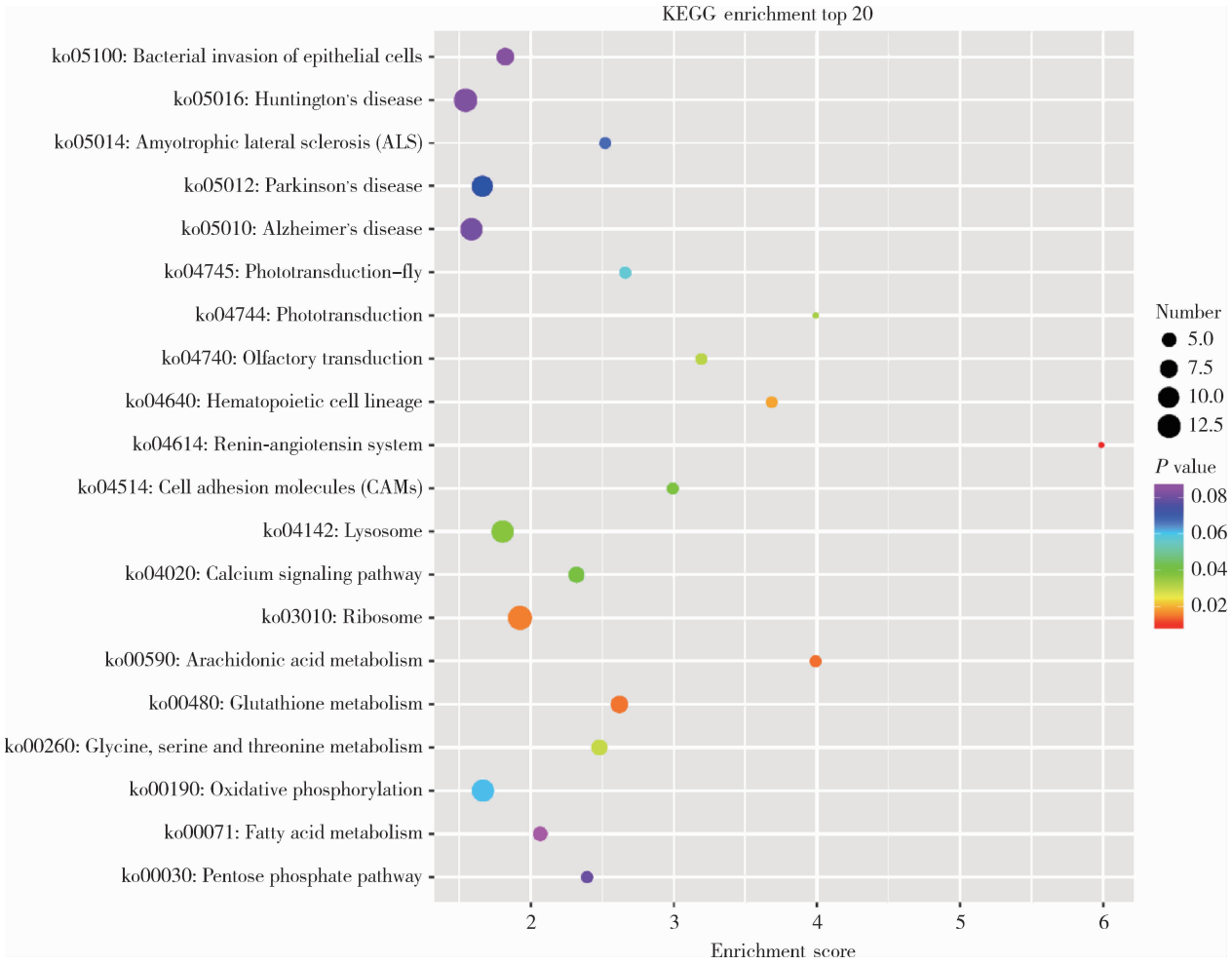

Fig. 8　Enriched DEPs pathways (1st instar *vs* 3rd instar) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

pathways, ribosome pathways, and lysosome metabolism, indicating that there was a lot of differentiation between the two groups in individual organ development and the genetic material of the translation process; thus, we inferred that the development of the 3rd instar larvae might have a certain degree of differentiation. Previous research has shown that in social insects pleomorphism is mainly related to differential gene expression (Barchuk *et al.*, 2007). Differences at the gene level will eventually be reflected at the protein level (Ribeiro *et al.*, 1999; Evans and Wheeler, 2000). Moreover, if grafting is carried out early, ovaries develop better, and more proteins are needed to support the high reproductive capacity. Several studies (Weaver, 1957, 1966) have shown that in late instars, larvae tend to develop into worker bees. When the larvae were > 4 d, only workers developed and bidirectional developmental potential was no longer observed. Research on queen ovary proteomics in different grafted instar larvae has indicated that queens developing from the larvae grafted at the 3rd instar have more proteins related to development, compared with queens that developed from the 1st instar grafted larvae. Studies on queens and workers at the gene and protein levels have shown that there were more up-regulated metabolism-related genes and proteins in queen larvae; however, there were more up-regulated development-related genes and proteins in worker larvae (Corona *et al.*, 1999; Li *et al.*, 2009). These genes could regulate the body's metabolic level to guide caste differentiation (Barchuk *et al.*, 2007). Obviously, the results of DEPs pathway enrichment were consistent with these studies.

Among the DEPs, hexamerins belong to down-regulated proteins. Hexamerins play an important role in insect metamorphosis and development. Hexamerins are synthesized by larval fat body, before the initiation of metamorphosis; they are progressively stored in the larval insect hemolymph, and released into fat body again at the end of larval development (Burmester and Scheller, 1999). Hexamerins are considered as a source of amino acids during pupal development. However, hexamerins may also play a role in caste differentiation (Martins *et al.*, 2008). Hexamerin 110

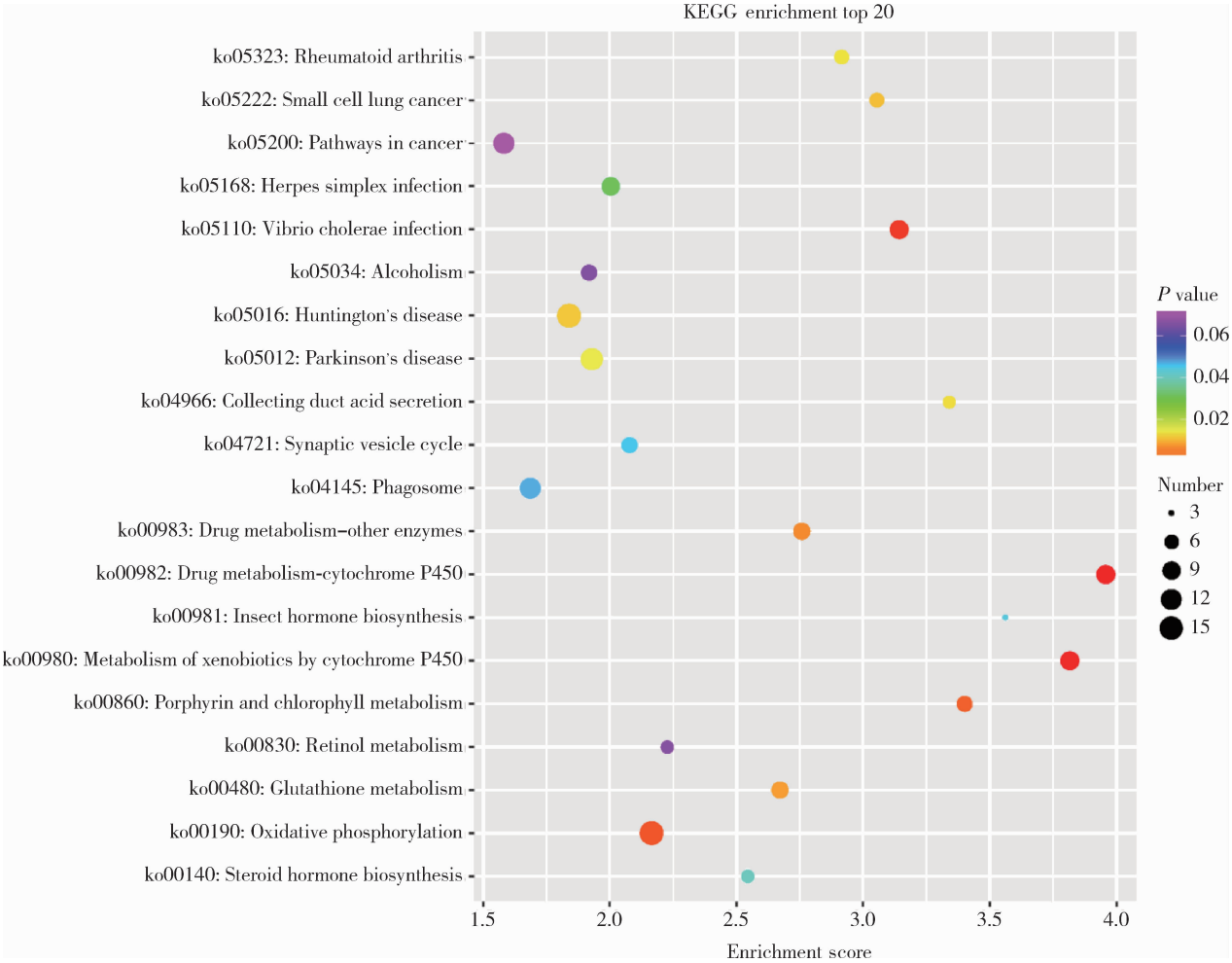

Fig. 9 Enriched DEPs pathways (2 instar *vs* 3rd instar) in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars

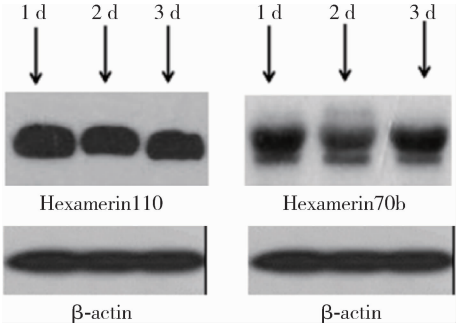

Fig. 10 Expression levels of hexamerin 110 and hexamerin 70b in the ovaries of *Apis mellifera* queens reared from the larvae grafted at different instars detected by Western blotting

1d: Queens reared from the 1st instar grafted larvae; 2d: Queens reared from the 2nd instar grafted larvae; 3d: Queens reared from the 3rd instar grafted larvae.

and hexamerin 70b transcripts are present in developing ovaries and testes. And hexamerin 110 not only participates in egg production by female insects, but also plays a role in metamorphosis and pupal development (Wheeler and Buck, 1995; Pan and Telfer, 1996, 2001; Seo *et al.*, 1998; Wheeler *et al.*, 2000), and hexamerin 110 is highly transcribed in the ovaries of egg laying queens (Martins *et al.*, 2010).

Additionally, both proteins are regulated by juvenile hormone and ecdysteroids (Braun and Wyatt, 1996; Cunha *et al.*, 2005). The study showed that juvenile hormone played an important role in caste differentiation of honeybees (Wirtz and Beetsma, 1972). Meanwhile, juvenile hormone can contribute to the ovary development during larval stage (Barchuk *et al.*, 2007), juvenile hormone and ecdysteroids have a synergistic effect on the development and metamorphosis of honeybees. In this study, the expression levels of hexamerin 110 and hexamerin 70b decreased with the grafted larval instar. Previous studies showed that the ovary development needs hexamerin 110 at a high expression level (Bitondi *et al.*, 2006), and juvenile hormone and ecdysteroids decreased with grafted larval instar (Pang *et al.*, 2017), and the results of expression levels of hexamerin 110 and hexamerin 70b in this study are consistent with these studies. We thus deduced that videlicet grafting instar earlier is beneficial to queen bee development.

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不同龄期移虫发育的蜂王卵巢蛋白质组学分析

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摘要：【目的】探讨移虫龄期对西方蜜蜂 *Apis mellifera* 蜂王卵巢发育的分子调控机制。【方法】运用相对和绝对定量同位素标记（iTRAQ）技术对不同龄期移虫培育的蜂王卵巢组织蛋白质进行定量分析，筛选差异表达蛋白。利用蛋白质免疫印迹法对结果进行验证。【结果】iTRAQ 定量分析共鉴定到蜂王卵巢组织蛋白质二级图谱 452 966 个，最终获得 3 642 个蛋白。GO（Gene Ontology）富集结果表明，不同龄期移虫发育的蜂王卵巢组织差异表达蛋白主要富集在细胞代谢、分裂以及蛋白质合成。Pathway 富集分析表明，1 龄幼虫期移虫和 2 龄幼虫期移虫发育的蜂王卵巢组织差异表达蛋白主要富集在碳水化合物代谢、脂代谢和外源降解类群；1 龄幼虫期移虫和 3 龄幼虫期移虫发育蜂王卵巢组织差异蛋白主要富集在有机体发育通路、核糖体通路和溶酶体代谢。与此同时，两个差异表达蛋白即储存蛋白 Hexamerin110 和 Hexamerin70b 的蛋白免疫印迹检测结果表明，随着移虫龄期的增加，Hexamerin110 和 Hexamerin70b 的表达量均呈现降低的趋势。【结论】对不同龄期移虫的蜂王之间差异表达蛋白进行鉴定，为进一步研究蜂王生殖发育及级型分化的调控机制提供理论依据。

关键词： 西方蜜蜂；移虫；龄期；卵巢；蜂王；蛋白质组学；差异表达蛋白

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